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(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
25 January 2001 (25.01.2001)

PCT

(10) International Publication Number
WO 01/06005 A2

- (51) International Patent Classification⁷: C12Q 1/68, (74) Agents: DEAN, John, Paul et al.; Withers & Rogers, Goldings House, 2, Hays Lane, London SE1 2HW (GB).
C07K 14/47
- (21) International Application Number: PCT/GB00/02741 (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (22) International Filing Date: 17 July 2000 (17.07.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
9916669.6 15 July 1999 (15.07.1999) GB
9926293.3 5 November 1999 (05.11.1999) GB
- (71) Applicant (*for all designated States except US*): THE UNIVERSITY OF BRISTOL [GB/GB]; Senate House, Tyndall Avenue, Bristol BS8 1TH (GB).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): MALIK, Karim [GB/GB]; Clic Unit, Dept. of Pathology, School of Medical Sciences, University Walk, Bristol BS8 1TD (GB). BROWN, Keith [GB/GB]; Clic Unit, Dept. of Pathology, School of Medical Sciences, University Walk, Bristol BS8 1TD (GB).
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— Without international search report and to be republished upon receipt of that report.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: DIAGNOSTIC METHOD

(57) Abstract: This invention provides a method of detection of cancer in a subject by detecting altered genomic imprinting and a method determining the long term prognosis of a subject diagnosed with cancer, using the differential methylation state of a specific nucleotide sequence to predict the long term prognosis.

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Diagnostic Method

This invention relates to a diagnostic method, to a nucleotide sequence comprising a Wilms' tumour suppressor gene (WT1) antisense regulatory region, and to a method of disease detection and prognosis based on the methylation state of the regulatory region.

Wilms' tumour (WT) is a childhood embryonal kidney tumour arising from the malignant transformation of renal stem cells. WT occurs in about 1 in 10,000 children, making it one of the commonest solid childhood tumours.

The human WT1 gene resides on chromosome 11p13 (Call *et al*, (1990) *Cell* 60, p 509-520; Gessler *et al*, (1990) *Nature* 343, p774-778; Call *et al*, (1994), US5,350,840) and is genomically organised as 10 exons spanning a 60 kilobase chromosomal region. Intragenic deletions and mutations of the tumour suppressor gene, WT1, have been detected in approximately 10% of Wilms' tumours.

During nephrogenesis i.e. kidney development, WT1 gene expression is controlled in a highly specific manner, increasing as metanephric mesenchymal cells progress towards immature epithelial cells, and attenuating as the cells become more phenotypically mature. The inverse correlation between WT1 expression and the differentiation status of human leukaemic cells along with evidence of expression in ovary and testis and the spinal chord and brain strongly suggest that the function of the WT1 gene product may be pivotal in growth and/or differentiation in a variety of cell types. The WT1 protein, which includes four zinc fingers, is expressed as four isoforms arising from two alternative splice sites (I and II) in the gene. Splice II occurs within the zinc finger domain, inserting or omitting three amino-acids (KTS) between zinc fingers 3 and 4. The WT1 protein without KTS amino acids (WT1-KTS) specifically binds to the EGR site consensus sequence (5'-GCGGGGGCG-3') whereas the WT1 protein with KTS (WT1+KTS) does not. By binding to the early growth response gene (EGR) type site(s) in the promoter regions of genes such as insulin-like growth factor type II (IGF-II), platelet derived growth factor A (PDGF-A), colony stimulating factor-1 (CSF-1), and epidermal growth factor receptor

(EGF-R) WT1 acts as a transcriptional repressor (reviewed in Hastie (1994) *Ann. Rev. Genet* 28, 523-558, and Menke *et al* (1998) *Int. Rev. Cytol.* 181, 151-212).

The human WT1 promoter region has been characterised and found to belong to the family of TATA-less, CCAAT-less, GC-rich promoters with multiple responsive sites for the transcription factor Sp1. EGR/WT1 consensus sequences were also identified upstream and downstream of the major transcriptional start site (Hofmann *et al.*, (1993) *Oncogene* 8, 3123-3132) and the suggestion that these sites may allow WT1 autorepression was subsequently verified using transient transfection assays with the human promoter (Malik *et al.*, (1994) *FEBS Letters* 349, 75-78)

WT1 function is crucial in the normal development of the urogenital system, as demonstrated in WAGR (Wilms tumour, Aniridia, Genitourinary abnormalities and mental Retardation) syndrome and in Denys-Drash syndrome (DDS), diseases characterised by renal and genital abnormalities together with a predisposition to Wilms' tumour (reviewed in Coppes *et al.*, (1993) *FASEB J.* 7, 886-895.)

The evidence for the involvement of WT1 in non-renal tissue differentiation is accumulating. A role in haematopoiesis is suggested by the downregulation of WT1 expression during chemically induced differentiation of HL60 cells (Sekiya *et al.*, (1994) *Blood* 83, 1876-1882) and K562 cells (Phelan *et al.*, (1994) *Cell Growth Differ.* 5, 677-686) Elevated WT1 expression in leukaemic cells relative to normal haematopoietic progenitor cells (Inoue *et al.*, (1997) *Blood* 89, 1405-1412) and the detection of WT1 mutations in leukaemias (King-Underwood *et al.*, (1996) *Blood* 87, 2171-2179; King-Underwood and Pritchard-Jones, (1998) *Blood* 91, 2961-2968) strongly implicate the involvement of the WT1 gene in leukaemogenesis. Altered WT1 expression has also been shown in breast cancers (Silberstein *et al.*, (1997) *Proc. Natl. Acad. Sci. USA* 94, 8132-8137)

Furthermore, antisense WT1 mRNA transcripts with no apparent open reading frames have been detected in foetal kidney and WTs, suggesting a regulatory role for these mRNAs (Campbell *et al.*, (1994) *Oncogene* 9, 583-595; Eccles *et al.*, (1994) *Oncogene* 9,

2059-2063). One such function of these mRNAs may be the formation of RNA heteroduplexes with sense WT1 mRNA, thereby modulating the finite levels of cellular WT1 protein. Previously the inventors reported the identification of an antisense WT1 promoter located in intron 1 which is activated by WT1. This effect of WT1 is reciprocal to that observed on the WT1 promoter, suggesting that the antisense promoter activity is involved in WT1 gene regulation (Malik *et al.*, (1995) *Oncogene* 11, 1589-1595). In addition, it has been demonstrated that expression of ectopic exon 1 RNA can affect the cellular levels of WT1 in an *in vitro* system (Malik *et al.*, (1995) *Oncogene* 11, 1589-1595; Moorwood *et al.*, (1998) *J. Pathol* 185, 352-359), supporting a regulatory role for antisense WT1 RNAs.

The WT1 antisense transcript may upregulate the levels of WT1 protein (Moorwood *et al.*, (1998) *J. Pathol* 185, 352-359), and aberrations of the control mechanisms for antisense RNA transcription may result in inappropriate temporal and spatial expression of WT1 protein, in turn contributing to tumourigenesis. In this regard, it is interesting to note that WT1 can increase the tumour growth rate of adenovirus-transformed baby rat kidney cells (Menke *et al.*, (1996) *Oncogene* 12, 537-546). The association between epigenetic modification of WT1 antisense regulatory regions, WT1 overexpression and renal tumourigenesis remains unclear, but preliminary studies have indicated that there is a correlation between hypermethylation of WT1 antisense regulatory regions and low WT1 protein, and the converse for hypomethylation. Interestingly, the WT1 antisense promoter locus was identified as a hypermethylated sequence in human breast cancers (Huang *et al.*, (1996) *Cancer Res.* 57, 1030-1034) and breast cancers have been shown to have decreased expression of WT1 (Silberstein *et al.*, (1997) *Proc. Natl. Acad. Sci. USA* 94, 8132-8137).

The inventors have identified an antisense regulatory region (ARR) of the WT1 antisense promoter, and have demonstrated that the ARR is part of a differentially methylated region. The WT1 ARR characterised and utilized as the basis of the invention is structurally and functionally distinct from previously described WT1 gene sequences (for example Call *et al.*, (1994), US patent 5,350,840). In addition, the inventors have found a correlation between the levels of ARR methylation, and the pathological state of human cells,

Specifically, a variety of cancer cells are shown to differ from their normal counterparts on the basis of epigenetic changes.

Accordingly, a first aspect of the invention provides a nucleotide sequence encoding a WT1 antisense regulatory region comprising at least a portion of, or consisting of, the sequence shown in SEQ1, or at least a portion of a variant, due to base substitutions, deletions and/or additions, of the sequence shown in SEQ.1.

A second aspect of the invention provides a nucleotide sequence encoding a WT1 antisense regulatory region comprising or consisting of the sequence shown in SEQ2, or at least a portion of a variant, due to base substitutions, deletions and/or additions, of the sequence shown in SEQ.2. The WT1 antisense regulatory region may be limited to the portion of sequence shown in bold in SEQ. 2, or variants of such a sequence due to base substitutions, deletions and/or additions.

A third aspect of the invention provides a nucleotide sequence encoding a WT1 antisense regulatory region negative regulatory element (NRE) comprising at least a portion of the sequence shown in SEQ.1 or at least a portion of a variant, due to base substitutions, deletions, and/or additions, of the sequence shown in SEQ.1. The nucleotide sequence shown in SEQ.1 may contain several *WT1* antisense regulatory region negative regulatory elements.

Preferably, a nucleotide sequence according to the first, second or third aspects of the invention is a DNA or RNA sequence. Portions of any sequences are preferably functional i.e. they have a biological function of a corresponding native sequence.

A fourth aspect of the invention provides a method of disease detection, diagnosis or prognosis in a subject with cancer, using the differentially methylated state of specific nucleotide sequences, such as the nucleotide sequences in the WT1 ARR region. Genomic epigenetic changes are often regional, for example affecting a variety of gene loci on chromosome 11p15 (Feinberg (1999) Cancer Research (suppl.) 59, p 1743-1746). The inventors' identification of the chromosome 11p13 region as a target for epigenetic changes

by methylation therefore suggest that other DNA probes/DNA sequences from the 11p13 region, including those derived from the 11p13 genes reticulocalbin and PAX6, may also be utilized for detection purposes in methods according to the invention.

The specific nucleotide sequence(s) may be one or more regulatory elements preferably one or more negative regulatory elements (NRE), for example, one or more NREs within the ARR. The NRE sequence or sequences may be part of the WT1 gene, or part of the chromosome 11p13 region, such that a method of disease diagnosis and prognosis in a subject diagnosed with cancer, comprises determining the methylation state of a NRE, or an ARR, of the WT1 gene or chromosome 11p13 region DNA sequence in the subject, and correlating the methylation state of the NRE with the diagnosis and expected long-term recovery prognosis of the subject. For example, in the case of acute myeloid leukaemias (AMLs), hypermethylation of the NRE indicates that the subject has a positive long term recovery prognosis, and hypomethylation of the NRE indicates that the subject is predisposed to relapsing after treatment. In the case of Wilms tumours, hypermethylation of the NRE indicates that the subject has a positive long term recovery prognosis, and hypomethylation of the NRE indicates that the subject is predisposed to relapsing after treatment. In Wilms' tumours, hypomethylation is detected specifically in tumours, and in colorectal cancer cell lines, hypomethylation correlates with tumourigenic potential. However, in other cancers, hypermethylation of the specific nucleotide sequence or sequences may indicate the presence of cancer cells and/or a predisposition of the subject to relapsing after treatment, whereas hypomethylation of the specific nucleotide sequence or sequences may indicate the absence of cancer cells and/or that the subject has a positive long term recovery prognosis. For example, see figure 1(e). The diagnostic application is underlined by the hypomethylation in WTs, as opposed to the hypermethylation of other renal tumours, such as primitive neuroectodermal tumour (PNET) and clear cell sarcoma of the kidney (CCSK) (see figure 1D).

The methylation state may be determined by restriction of the WT1 antisense regulatory region using enzymes such as *Bsh1236I*, *SpeI* and *KpnI* in combination. *Bsh1236I* is an isoschizomer of *Bst* UI. *Bsh1236I* cuts at the restriction sequence CGCG only when there is no CpG methylation. Methylated sequences are not restricted by *Bsh1236I*. Therefore,

the restriction pattern obtained for a nucleotide sequence which has been restricted with Bsh1236I gives a different band pattern depending on whether the Bsh1236I sites in the nucleotide sequence are methylated or not. Other commercially available enzymes may also be used, with one or more being able to distinguish between methylated and unmethylated DNA.

The methylation state may be determined using a PCR-based assay system. Such a PCR-based assay system may involve the use of sodium-metabisulphite. This has the effect of converting all unmethylated cytosine residues to uracil residues. Preferably, the PCR reaction uses the following primers to amplify at least a portion of the WT1 antisense regulatory region:

Tf: 5'-GGGTGGAGAAGAAGGATATATTTAT-3'.

Tr: 5'-TAAATATCAAATTAATTTCTCATCC-3'.

TfN: 5'-GATATATTTATTTATTAGTTTTGGT-3' (nested primer).

TrN: 5'-AAACCCCTATAATTTACCCTCTTC-3' (nested primer).

The conditions used in the PCR reaction are the same as the conditions mentioned later in the specification. The PCR products obtained from the PCR reaction, as described below, may then be cloned and sequenced. The PCR products may be cloned into a vector such as pGEM-T (Promega). Alternatively, the PCR products may be sequenced directly. Once sequenced, any methylated cytosine residues will remain readable as 'C' in the nucleotide sequence, whereas unmethylated cytosines will appear as 'T' residues in the sequence.

The nested PCR reaction involves the following primers.

Tf: 5'-GGGTGGAGAAGAAGGATATATTTAT-3'.

Tr: 5'-TAAATATCAAATTAATTTCTCATCC-3'.

TfN: 5'-GATATATTTATTTATTAGTTTTGGT-3' (nested primer).

TrN: 5'-AAACCCCTATAATTTACCCTCTTC-3' (nested primer).

A fifth aspect of the invention provides a method of cancer detection in cells derived from a subject comprising detection of tumour-specific alteration of genomic imprinting. Any bi-allelic expression of tumour-specific genes may indicate the presence of tumourigenic cell proliferation if the normal tissue expresses the gene monoallelically. Alternatively, with some cancers, the normal tissue may be biallelic, and the cancer monoallelic. Additionally, methylation changes may be accompanied by changes in gene expression through silencing or enhanced gene expression, irrespective of allelic contributions to gene dosage (reviewed in Jones (1996), Cancer Research 56, p2463-2467)

The tumour-specific alteration of genomic imprinting may be detected by reverse transcription PCR (RT-PCR). This allows relatively fast detection of altered genomic imprinting by visual analysis of the RT-PCR products on an electrophoretic gel.

The method may be used in the detection of WT in a subject, and may detect alteration of genomic imprinting of WT-specific genes such as the WT-1 gene.

The altered genomic imprinting detected may be relaxation of genomic imprinting, loss of imprinting, or gain of imprinting.

The RT-PCR may use two primers designed to anneal to a tumour-specific gene sequence on opposite sides of an allelic polymorphism which introduces a restriction-site in one allele only. For example, in the case of WT, the RT-PCR may use the following primers:

Primer 1: WT18 [CTTAGCACTTTCTTCTTGGC]

Primer 2: WITKBF2 [TTGCTCAGTGATTGACCAGG]

A sixth aspect of the invention provides a method of treating a subject with a specific cancer, comprising altering the genomic imprinting of a tumour-specific gene. This may involve relaxation of the genomic imprinting, or reversal of relaxed genomic imprinting.

A seventh aspect of the invention provides a diagnostic kit, assay or monitoring method using a method according to a fifth aspect of the invention.

An eighth aspect of the invention provides a method of detection of the methylation state of a WT1 antisense regulatory region comprising detection of a tumour-specific alteration in genomic imprinting using a method according to a preceding aspect of the invention, and correlating a detected alteration in genomic imprinting with differential methylation of the WT1 antisense regulatory region. For example, relaxation of genomic imprinting may be correlated with hypomethylation of the WT1 antisense regulatory region.

Nucleotide sequences, and methods of disease diagnosis, detection and prognosis in accordance with the invention will now be described, by way of example only, with reference to accompanying Figures 1(A) to 3(B), and SEQ.1 to SEQ. 3 in which;

Figure 1(A) shows the probe used for the detection of methylation for Southern blotting; and

Figure 1(B) shows a Southern blot of three acute myelogenous leukaemia (AML) DNAs and a normal peripheral blood lymphocyte DNA; and

Figure 1(C) shows a Southern blot of DNAs from a non-tumourigenic and a highly-tumourigenic colorectal cell line; and

Figure 1(D) shows a Southern blot of matched normal kidney and WT samples, matched normal kidney and PNET or CCSK DNAs and a foetal kidney control; and

Figure 1(E) shows Southern blot analysis of breast tumour DNAs for changes in the ARR methylation status.

Figure 2 shows the nucleotide sequence of a WT1 ARR, with the primer hybridisation sites indicated by arrows; and

Figure 3(A) is a schematic diagram showing the primers on either side of the antisense WT1 RNA splice used for RT-PCR; and

Figure 3(B) shows a southern blot of the antisense WT1 RNA RT-PCR products; and

SEQ.1 shows a nucleotide sequence of the WT1 ARR; and

SEQ.2 shows a nucleotide sequence of a negative regulatory element of a gene encoding WT-1; and

SEQ.3 shows the nucleotide sequence of a WT1 antisense region (Gessler, M & Bruns (1993) Genomics 17: 499-501) with the RT-PCR primers shown as arrows and the exonic sequences indicated in bold.

1. Cloning and characterisation of WT1 genomic sequences

The WT1 cDNA and WT1 promoter region were cloned from a human foetal kidney cDNA library (Clontech) and a human B-cell genomic library (λ Sh2001, kindly supplied by T. H. Rabbitts, Medical Research Council, Cambridge) respectively. For each library, Plaque screen filters (Du Pont) were prepared *in situ* from 1×10^6 phage (Benton, W. D. and Davis, R. W. (1977). *Science*, **196**, 180-182). Filters were hybridized in 6x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5x Denhardt's solution, 0.5% SDS and 100 μ g/ml salmon sperm DNA at 65°C. Washing was performed at high stringency (0.1x SSC, 0.5% SDS, 65°C). For the cDNA library, a partial WT1 cDNA obtained by PCR amplification was used as probe. The DNA sequence of a full-length cDNA isolated from the cDNA library was determined by the dideoxy chain terminator method (Sanger, F., *et al* (1977). *Proc. Natl. Sci. USA*, **74**, 5463-5467), and a 700 bp fragment from the 5' terminus of the cDNA was used for probing the genomic library. Probes were radiolabelled with [α - 32 P]dCTP (Amersham) according to the random primer method (Feinberg, A. P. and Vogelstein, B. (1983). *Biochem. Biophys. Res. Commun.*, **111**, 47-54).

Genomic clones corresponding to the 5'-end of the WT1 gene were subcloned and characterised by restriction analysis according to standard methodology (Sambrook, J., *et al* (1989). *Molecular Cloning, Vols 1 and 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*). DNA sequences were determined by the dideoxy chain terminator method (Sanger, F., *et al* (1977). *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467) and by Δ taq cycle-sequencing according to the manufacturers instructions (USB-Amersham). The functional assessment of DNA from intron 1 of the WT1 gene was carried out by transient transfection of reporter gene constructs with various WT1 intronic sequences directing gene expression (Malik, K., *et al* (1995) *Oncogene*, **11**, 1589-1595).

2. Differential Methylation assays

Human genomic DNAs are purified by standard phenol-chloroform extraction procedures (Sambrook, J., *et al* (1989). *Molecular Cloning, Vols 1 and 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*). Based on the DNA sequence of the intronic region (see Figure 2), digestion by restriction enzyme Bsh1236I (MBI Fermentas) has been

selected to examine methylation of the intronic region. This enzyme cuts at the restriction sequence CGCG only when there is no CpG methylation; methylated sequences are not restricted. Our work has established that differential methylation is conveniently detected within a KpnI - SpeI (New England Biolabs) fragment of 850 bp, which contains 4 potential Bsh1236I sites (see Figure 1) . Depending on whether these sites are methylated or unmethylated, a characteristic banding pattern is observed after digestion of genomic DNAs with a combination of KpnI, SpeI, and Bsh1236I, Southern blotting and hybridisation with a radiolabelled DNA probe (Sambrook, J., *et al* (1989). *Molecular Cloning, Vols 1 and 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*) defined by the KpnI and SpeI sites in the intronic sequence (Figures 1 and 2).

Figure 1(D) shows a Southern blot of matched normal kidney and Wilms' tumour samples. All WT samples were confirmed as having no loss of heterozygosity. Also shown are matched normal kidney and PNET or CCSK DNAs.

As shown in Figure 1 (D), the pattern of differential methylation successfully distinguishes between normal kidney DNA and Wilms' tumour DNA (panel A), leukaemic cells from patients with varying prognosis and normal lymphocytes (panel B) and also highly tumourigenic and non-tumourigenic colonic cell-lines (panel C). The results shown in panel C suggest that this change may be associated with the tumourigenic process and may therefore be relevant to cancers other than only Wilms' tumour.

In Wilms' tumours, hypomethylation of specific nucleotide sequences correlates with the tumour state. However, in other cancers, this correlation may be inverted, such that hypermethylation of specific nucleotide sequences corresponds to the methylation status of tumour cells, and hypomethylation may indicate normal cells. An example of this is shown in figure 1(E) , with Southern blot analysis of normal breast tissue DNA and breast tumour DNAs for changes in the ARR methylation status. Four infiltrating ductal carcinomas of varying aggressiveness all showed increased methylation of the WT1 ARR compared to the normal breast tumour DNA. Therefore the relative differential methylation comparing normal tissue and tumour tissue may be utilised diagnostically.

3. PCR-based assay system

Tumour cells and normal cells may be distinguished by their epigenotype as previously outlined. Knowledge of the DNA sequence of the WT1 antisense regulatory region has made it possible to develop a PCR-based assay system to allow the determination of the methylation status of samples, which will require less biological material. This method involves introducing CpG dinucleotides which are not part of a restriction enzyme recognition sequence by treatment of genomic DNA samples with sodium-metabisulphite (Merck), thereby converting all unmethylated cytosine residues to uracil (Paulin, R., *et al* (1998) *Nucleic Acids Research* 8, 4777-4790). Specific regions of interest in the WT1 intronic sequence can then be amplified using primers specific for both strands of DNA. The PCR bands obtained can be directly sequenced or cloned using a commercially available vector such as pGEM-T (Promega) and analysed by DNA sequencing. Any methylated cytosine residues will remain readable as 'C' in the DNA sequence, whereas unmethylated cytosines will appear as 'T'.

Alternatively, after the first round of PCR on bisulphite-treated DNA, nested primers which include one specific for the methylated Bsh1236I site shown to be commonly differentially methylated (boxed in Figure 2), or one specific for the unmethylated Bsh1236I site (i.e. specific for C→T conversion) may be employed, permitting discrimination between methylated and non-methylated sequences by visualisation of the PCR products, i.e. if a primer specific for the methylated Bsh1236I site is used, a PCR product will only be observed if the Bsh1236I site in the sample is methylated, otherwise, no PCR amplification will occur.

Illustrative primers which may be used for methylation-specific PCR are shown below, and their hybridisation positions to the WT1 sequence are shown by arrows in Figure 2 for top-strand amplification. Allowing for C→T conversion, these are:

Tf: 5'-GGGTGGAGAAGAAGGATATATTTAT-3'.

Tr: 5'-TAAATATCAAATTAATTTCTCATCC-3'.

TfN: 5'-GATATATTTATTTATTAGTTTTGGT-3' (nested primer).

TrN: 5'-AAACCCCTATAATTTACCCTCTTC-3' (nested primer).

Typical primary amplifications are conducted with Amplitaq (Perkin-Elmer) with 100 ng. of bisulphite-treated DNA in buffer supplemented with 3mM MgCl₂. Amplification conditions are 3 mins. denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 30 secs, annealing at 50°C for 30 secs, and extension at 72°C for 90 secs. A final extension of 5 mins at 72°C completes the reaction. Secondary PCR with the nested primers employs the same conditions, but using 1/100th of the primary PCR reaction and 24 cycles.

4. Correlation of the methylation state of the (NRE) with long term disease prognosis

The inventors have detected a correlation between the methylation state of the ARR and the diagnosis and long term disease prognosis in subjects with cancer. The diagnostic potential is shown by the hypomethylation in WTs, as opposed to the hypermethylation of other renal tumours, such as primitive neuroectodermal tumour (PNET) and clear cell sarcoma of the kidney (CCSK) (see figure 1D). AML subjects with hypermethylated ARR, responded well to treatment and made a full recovery. However, subjects who had an unmethylated NRE, and relapsed, were refractory to treatment.

Therefore, the methylation state of the NRE can be used as a potential early indicator of the long term diseased prognosis. Subjects who have an unmethylated NRE can be kept under closer observation for early detection of relapse. This will maximise their chances for recovery. However, the expense of such close observation post-treatment is not necessary with subjects with unmethylated NRE, as these patients are expected to respond well to treatment once any relapse has been detected by normal routine checking.

In pilot studies with AMLs, hypermethylation of specific nucleotide sequences corresponds to a predicted positive long term prognosis of the subject with the AML, and hypomethylation corresponds to a predisposition of the subject to relapsing after treatment. However, in other cancers, this correlation may be inverted, such that hypermethylation of specific nucleotide sequences corresponds with a predisposition to relapsing after treatment, and hypomethylation may indicate a positive long term prognosis for recovery.

Therefore, decisions on the best methods of therapy to suit the subject can be made in the light of an educated expectation of how the subject is expected to respond to treatment in the event of a relapse of their cancer condition.

Therefore, it is the differential methylation that is the determinant in developing long term prognosis for subjects diagnosed with cancer.

5. Genomic imprinting of the WT1 gene

The WT1 allele specific methylation pattern observed in normal kidney cells strongly indicates that there is genomic imprinting of the WT1 ARR/NRE (Antisense Regulatory Region/Negative Regulatory Region) and tumour-specific relaxation of genomic imprinting in Wilms' tumours.

Genomic imprinting is the phenomenon by which maternal or paternal copies of a gene can be selectively expressed, with methylation of DNA serving as the regulatory signal. Loss of such a signal can lead to an altered dosage of gene expression that can be deleterious to normal cell growth. For example, the *IGF2* gene exhibits loss of genomic imprinting control of *IGF2* and is overexpressed in WTs (Feinberg, A. P. (1999) *Cancer Res. (suppl.)*, 59: 1743s-1746s). As *IGF2* is a growth factor, this may easily contribute to uncontrolled proliferation associated with tumourigenesis.

In order to determine whether the differential methylation of the *WT1* ARR/NRE is accompanied by allele specific expression of the WT1 antisense RNA (*WT1-AS*), reverse transcription-PCR (RT-PCR) analysis was conducted on foetal and normal kidney cells, and WT cells. Primers either side of the antisense *WT1* RNA splice (see SEQ3 and Figure 3A) (Gessler, M., and Bruns (1993), *Genomics*, 17: 499-501, 1993) were used for RT-PCR:

Primer 1: WT18 [CTTAGCACTTTCTTCTTGGC]

Primer 2: WTKBF2 [TTGCTCAGTGATTGACCAGG].

Typical reaction conditions used for the RT-PCR were annealing of the reverse primer to 1 µg of total RNA by heating to 60°C for 5 mins, followed by quenching on ice, followed by reverse transcription carried out with Super RT (HT Biotechnologies, Cambridge, U.K.)

reverse transcriptase at 50°C for 60 mins. This was followed by PCR cycling as follows:

95°C, 3 mins. (1 cycle);

94°C, 15 secs., 60°C, 30 secs., 72°C, 60 secs. (2 cycles);

94°C, 15 secs., 58°C, 30 secs., 72°C, 60 secs. (2 cycles);

94°C, 15 secs., 56°C, 30 secs., 72°C, 60 secs. (10 cycles, 20 for antisense product); and

94°C, 15 secs., 56°C, 30 secs., 72°C, 60 secs. with 20 secs. extension per cycle (20 cycles).

The PCR products obtained were digested by adding the restriction enzyme *MnII* directly to the PCR mix and incubating for 60 minutes at 37°C. The PCR products were then separated on 2% agarose gels and then alkali blotted onto Hybond N⁺ membrane and hybridised with a ³²P-labelled antisense cDNA probe. The sequence of the probe is shown in bold between WT18 and WITKBP2 in SEQ. 3. The following primers were used as DNA controls:

Primer 1: WITKBF2 [TTGCTCAGTGATTGACCAGG]

Primer 2: WITKBR2 [TTGGCTGGAAAGCTTGCAGC]

The *MnII* polymorphism (Grubb, G. R. *et al* (1995) *Oncogene*, 10: 1677-1681) utilised is marked by an asterisk in figure 3A, and results in RT-PCR products of 286 **and** 222bp for biallelic expression, or alternatively major allelic bands of 286bp **or** 222bp for monoallelic expression.

As shown in figure 2B, expression of WT1-AS in normal kidney samples that have one methylated and one unmethylated allele, only occurs from one allele, confirming genomic imprinting. However, WTs display biallelic expression of WT1-AS, thus revealing a relaxation of imprinting control in WTs. The net increase arising from expression of both *WT1-AS* alleles may thus serve as an additional marker of the differential methylation pattern detected in Wilms' tumours.

This altered imprinting is likely to be present in cancers other than WT, and therefore, altered imprinting control of specific genes may provide a marker for the detection or

diagnosis of various cancer types in a patient. Furthermore, as epigenetic modifications of DNA are reversible, detection of altered imprinting control and/or the diagnosis of methylation changes should also facilitate therapeutic strategies based on enzymes such as DNA methyltransferases and demethylases, or by chemical compounds (Jones P.A. and Laird P.W. (1999), *Nature Genetics*, 21, p163-167). This would enable control of gene expression and permit therapies that are contingent on appropriate gene control.

SEQ.1

```
CTCGAGGATCCAGAGACGGCCTTGATCCTCTCCCCTGGGGTTTGGCCTTGGCGCTCTGAT
GGCCATTTCCACATTTTTGAGAGTTGATGCCCTTGCCTCTCACAGCCCAAGTCTTGGGCC
AGGCCCTGCATTCTGGGGAAGCAGCAGGAACCCTGGAAATCCAAAGAATAAACCCAGAA
TCTCGAGGGCCACCCTTGCCCACTCCAGGATAGCAGCCGGAGCGCTTCTCACATCCAAGC
TGCCCAATGAGCCTCAAGGGCTGGGTAAGATGGACCCATCTGTTTTCACTGCAAGACAAA
ACTTAAACCTGGAGATGGTGCTTCCAGGCTATATGACTTGAATCTAGGGCCCTCTCTCCA
TTGGGCTTTTTCTCCAGGGTGGAGAAGAAGGATACATTACCTACTAGTCCTGGTCCCCT
TTTAACTTTTTCTCCATGGCAGCCACGCCTGTATATTACAGAAGAATCCAGATATTTTCC
AGAAGTGTAATACCTGCTGGCTGCAAAACCCACAGTCCCACCCCCACGACATGTGATAA
GATCCCAGGCACCAGACCTGCCCTGAAAAGGGCTGGACAAGGGACCCAAACGAAGCGACA
GAACCCAGGTTTCAAAAATCCCTTAGAAGTACTAAAAAGATAATGGCGTAGTAGTATTTT
GTGCCCCAGGGGCATGGATTTCGATGGTTTCTCAACCGCCTCCAAATAGCACACATGCAGA
CAGTGCTCTCGGATTCATTGTTTCTCAGTCACAGATGTTTAGATGGGTTGCCGAGTTCCA
TATTTAAAGCCCCAAGAGGGTGGTGGGTAGCGCTTCTGCATCTATGGAGTATAACTTCAA
GCCGGACCCAATCTCCAGGTTGCCCATCTCAGCTGTCTCTTATAGACGGGGACACTGAG
ACCTAGAACTCCCCAAAAGTAACACCAGCCTGCTAAACAAAGGTGGCGCGATCTGATCA
AAGAACACAAGCCTCAGCGATTAGTAAGTTGTCCAACGCCCCTTGAGTAGAAACACTAAT
TTACTAACTAAAAGCATAGAGTGGAGGCTTCCCTTGGGTCTGCTTGCGGTTCCCTCCACAG
GACAGTGATCCCAGATTCTCCCGAAGAAAAGGGCGGTTTCGATTTCTCCAAGGCTTCGCG
GGGGCCGGGTGCTCCTGGTTAAACTAAGGTAGGAGCGGCCTGAAGACGCGCGTTTAGAAG
GCGCCGGGTGAAGGCGGGCAACAAGGCAGAGCCCTTCTCCGAGCCTTGGGCGAAGGTAC
CTCCTGCAAAAGATACACTCTGCTTCCCACGCATTCCAAAAACATCCCGGTCCCTAGGCC
CTCGAGTAATTTTGCTCCAGGAAAAGCATCCGCCATTGTATTAGTAAAGCGTTTACTAAA
TTACCGAATCAAACCGAACTGGCTTAGGTTCTCAATAGCGTGGAATCCACTGAAAATAA
ATGAAGAGGGCAAACTACAGGGGCTCCGCAGGTTCCGGTCCGCGCCGCCAGGCAGAAAGA
```

GAGGTGGGCGGGCATCGGCGCGGGATGAGAAACCAACCTGATACTTATCGTGTGCCGAGT
TCCCTCCTTGTATCCTGACTAAGCACAGCGAATAACCCTGTCCTTGTTCTAACCCAGGT
CTTGAAGAAATACTGTCCCAGCTGAGCCCCGCGTTTACAAGATGAAGAGGCGCCCCAGAT
GCGCTGAAAGAAAGGCCAAAGCTCGTGCCTCCTTCCACTGCCTGCGGTAGAACCTGGTCC
CGCATAGCTTGGAATCGGATAAGTCAAGTTCTCTTCCATCCCCAGAACCTGCGTGGCCGC
CGCCTGAGCGAAGCCCAGTGAAGATCCACTTCTGTATTACCATACGGGGG

SEQ.2

TTCTGCATCTATGGAGTATAACTTCAA
GCCGGACCCAATCTCCAGGTTGCCCATCTCAGCTGTCCTCTTATAGACGGGGACACTGAG
ACCTAGAAACTCCCCAAAAGTAACACCAGCCTGCTAAACAAAGGTGGCGCGATCTGATCA
AAGAACACAAGCCTCAGCGATTAGTAAGTTGTCCAACGCCCCCTTGAGTAGAAACACTAAT
TTACTAACTAAAAGCATAGAGTGGAGGCTTCCCTTGGGTCTGCTTGCGGTTCCCTCCACAG
GACAGTGATC

SEQ.3

1 TTCCTGTCGG GTCCCTGGGG TCCTCCGACT GCGGCTCCTC AGCTTAGCAC
51 TTTCTTCTTG GCCCCGCAGG CTGCAGGGAA CTCCTCCAC CTCTTTAGTC
→
WT18

101 GGAGAAGTCC AAGTCGGGCG AGGGGGCACC CCGGGGTTCG CACCGGTGCT
151 CTTCCCCTCC CCGCCCCAC AAGGATTCTG AGAAAATAAA TGGCAGAGGA
201 GAGAGGAGTT CTACATTGTC TTGGCTCTCC TTTCTCCTA TCCACCCCTA
251 CATCCCTCAC CCCGGNNCAA AAACCTATTT TTGAAAAATG TTGGCAGAGA
301 TTTACGTGTC TTTGCCTTAC CTGGGTTTCA CAAACACAAC GACTCACATT
351 CAAGCCAGCC TCCCTTCAGA TAACCTCCTC TCCCCCGCT AAAAGTGCCA
401 AGGATGGTAA AAGAAGAAAC AATCTCAATC TTTTCGTTT GAAATGAAAG
451 TCCCCGGCTT TTCATAAAGG GCTCCTCGCC CTCACAGTT GAGTCCTAGT
501 TAAGAAAAAC GACTTCCAAG TAGAAATAAT AGGCGGGGAG AAGGAAGGGA
551 GATACAGGGA TCTGGGGNGT TCTTAGGGCA ACTGGCAGTG AATTTTGTCT
601 CGAGAGTCCT TTCTCCACTC AAAAAACCAA ACGCGCGAGC CCCGCGAAAG
651 GTTTAGGGAT AGATCGTGTG GGAGAGGACT GAGCAGAGAG CGTGGGGGCA
701 GTGTCTTGTA GAATCTTTCT TTTCTTAATA ATAATTTTAA AAGCTTCTGA
751 GTGGAGACGA CGCAAAGTCA AGCAGCAAAG GTGGCCTGGG AGGCAAGCGG
801 AGGGCTCAAG TGCCGCATCT TTACCCTCAG GGTCTCCTGC GCCTACGGGA
851 TGCGCATTCC CAAGAAGTGC GCCCTTCGAG TAAGTCCTGG GCCCCACAC
901 ACTTCGGGTC CGCAGCCAGA ATTTAATGGC GACAACGTTT ATGCAATGCA
951 AGCTAAAAAC CAAAGCGTAA AAAATTACTA TGTCATTTAT TGAAACGCCA
1001 TTCTTTGTCA AACTGCAACT ACTTTGCTTC ACATAAGTTT GGCTGGAAAG
1051 CTTGCAGCCC CAGCCCGGGC CAGCCAGGTA CAGGAGGCCG GACTGCAACC
1101 GGTGCTTCC CTCCCGTCGC GCCTGGCCGT CCCACGCTGC GCCGTCGCTG
1151 CTGCCTCCTG GCGCCCCTGG GATTTTATAC GCACCTCTGA AACACGCTCC
1201 GCTCCGGCCC CCGGTTCTTC TCCTTGCTTA GGGGTTGTTT CCAATAGAT
1251 ACTGACTCCT TTAGAAGATC CAAAAACCAA ACCAAAACAC CCCCTACCCG

1301 CCCCAAACAC CTGCTCTGGG GCGCGGGGGC0 TGCCAAACAG AGACTAGACG

1351 AAGGGAGTCA GATTAGCGA AGCTCTTCGA GTCCTCAAAG ATTCGAACAC

1401 TAACTCGCGC CCGTGGGCCG ATGGAGGTTT TCCCTACTCC ACTCCTTGGT

1451 CCCCTTAACT GGCTTCCGCC TCCTGGTCAA TCACTGAGCA ACCAGAATGG

←
WITKBF2

1501 TATCCTCGAC CAGGGCCACA GGCAGTGCTC GCGGGAGTGG CTCCAGGAGT

1551 TACCCGCTCC CTGCCGGGCT TCGTATCCAA ACCCTCCCCT TCACCCCTCC

1601 TCCCCAAACT GGGCGCCAGG

Claims

1. A nucleotide sequence encoding a WT1 antisense regulatory region comprising at least a portion of the sequence shown in SEQ.1 or at least a portion of a variant, due to base substitutions, deletions, and/or additions, of the sequence shown in SEQ.1.
2. A nucleotide sequence according to claim 1 which encodes a WT1 antisense regulatory region negative regulatory element (NRE).
3. A WT1 antisense regulatory region negative regulatory element (NRE) comprising at least a portion of the nucleotide sequence shown in SEQ.2 or at least a portion of a variant, due to base substitutions, deletions, and/or additions, of the sequence shown in SEQ.2.
4. A WT1 antisense regulatory region NRE according to claim 3 wherein the NRE comprises the sequence shown in bold in SEQ. 2, or variants of such a sequence due to base substitutions, deletions and/or additions.
5. A nucleotide sequence or NRE according to any preceding claim wherein the nucleotide sequence is a DNA sequence.
6. An RNA sequence encoded by a nucleotide sequence according to any preceding claim.
7. A method of disease diagnosis and prognosis in a subject diagnosed with a Wilms' tumour cancer, the method comprising determining the differentially methylated state of a specific nucleotide sequence or sequences in the subject, or in a sample derived from the subject.
8. A method according to claim 7 wherein the specific nucleotide sequence or sequences form part of the WT1 antisense regulatory region (ARR).

9. A method according to claim 7 or claim 8, comprising determining the methylation state of a negative regulatory element (NRE) or an ARR of a WT1 gene in a sample isolated from the subject, and correlating the methylation state of the NRE or ARR with the diagnosis and expected long-term recovery prognosis of the subject.
10. A method according to claim 9 wherein hypermethylation of the NRE or ARR indicates that the subject has a positive long term recovery prognosis, and hypomethylation of the NRE or ARR indicating that the subject is predisposed to relapsing after treatment.
11. A method according to claim 7 or 8, wherein hypomethylation of the specific nucleotide sequence or sequences indicates that the subject has a positive long term recovery prognosis, and hypermethylation of the specific nucleotide sequence or sequences indicates that the subject is predisposed to relapsing after treatment.
12. A method according to any one claims 7 to 11 wherein the NRE is a nucleotide sequence according to any one of claims 1 to 6.
13. A method according to any one of claims 7 to 12 wherein the methylation state is detected by restriction digest analysis.
14. A method according to claim 13 wherein at least enzyme Bsh1236I is used to restrict the NRE.
15. A method according to any one of claims 7 to 12 wherein the methylation state is detected using a PCR-based assay system.
16. A method according to claim 15 wherein the PCR assay system uses at least one of the following primers to amplify a region of nucleotide sequence:

Tf: 5'-GGGTGGAGAAGAAGGATATATTTAT-3'.

Tr: 5'-TAAATATCAAATTAATTTCTCATCC-3'.

TfN: 5'-GATATATTTATTTATTAGTTTTGGT-3' (nested primer).

TrN: 5'-AAACCCCTATAATTTACCCTCTTC-3' (nested primer).

17. A method according to claim 16 wherein the amplified nucleotide sequence is cloned and sequenced.
18. A probe comprising a nucleotide sequence according to any one of claims 1 to 6.
19. A diagnostic kit, assay, or monitoring method using a nucleotide sequence according to any one of claims 1 to 6 or a probe according to claim 18.
20. A diagnostic kit, assay, or monitoring method using a method according to any one of claims 7 to 17.
21. A method of cancer detection in a subject or in a sample isolated from the subject comprising detection of the methylation state of a specific nucleotide sequence or sequences.
22. A method according to claim 21 comprising correlating the methylation state of the specific nucleotide sequence or sequences with the presence or absence of cancer cells in the subject.
23. A method according to claim 22 wherein hypomethylation of the specific nucleotide sequence or sequences indicates the presence of cancer cells in the subject.
24. A method of cancer detection in cells derived from a subject comprising detection of tumour-specific alteration of genomic imprinting.
25. A method according to claim 24 comprising the detection of tumour-specific relaxation of genomic imprinting by determining the methylation state of a specific nucleotide sequence.
26. A method according to claim 24 or claim 25 wherein the tumour-specific alteration of genomic imprinting is detected by reverse transcription-PCR (RT-PCR).

27. A method according to any one of claims 24 to 26 wherein the cancer is Wilms' Tumour (WT).
28. A method according to claim 27 comprising detection of the relaxation of genomic imprinting of the antisense WT-1 RNA sequence.
29. A method according to claim 28 wherein the RT-PCR uses two primers, designed to anneal to the tumour-specific gene sequence on opposite sides of an allelic polymorphism which introduces a restriction site in one allele only.
30. A method according to claim 29 wherein the RT-PCR uses the following primer pair
- Primer 1: WT18 CTTAGCACTTTCTTCTTGGC
- Primer 2: WITKBF2 TTGCTCAGTGATTGACCAGG.
31. A method of treating a subject with a specific cancer comprising altering the genomic imprinting of a tumour-specific gene.
32. A method according to claim 31 wherein the genomic imprinting of a tumour-specific gene is altered by altering the methylation state of a specific nucleotide sequence.
33. A method according to claim 31 or claim 32 wherein the genomic imprinting is altered to relax the genomic imprinting of the tumour-specific gene.
34. A method according to claim 31 or claim 32 wherein the genomic imprinting is altered to reverse the relaxation of the genomic imprinting of the tumour-specific gene.
35. A diagnostic kit, assay or a monitoring method using a method according to any one of claims 24 to 30.
36. A method of detection of the methylation state of a WT1 antisense regulatory region comprising detection of a tumour-specific alteration of genomic imprinting using a method according to any one of claims 21 to 30 and correlating a detected alteration in relaxed genomic imprinting with differential methylation of the WT1 antisense regulatory region.

37. A method according to claim 36 wherein the alteration in genomic imprinting is a relaxation in genomic imprinting.
38. Method of treatment comprising selecting a particular course of therapy on the basis of the results of a method according to any preceding claim.

FIG. 1

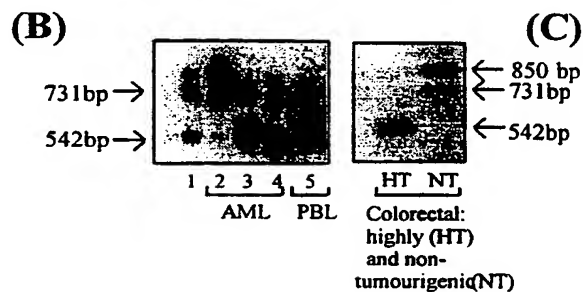
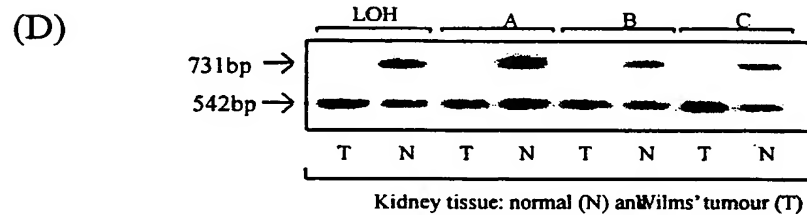
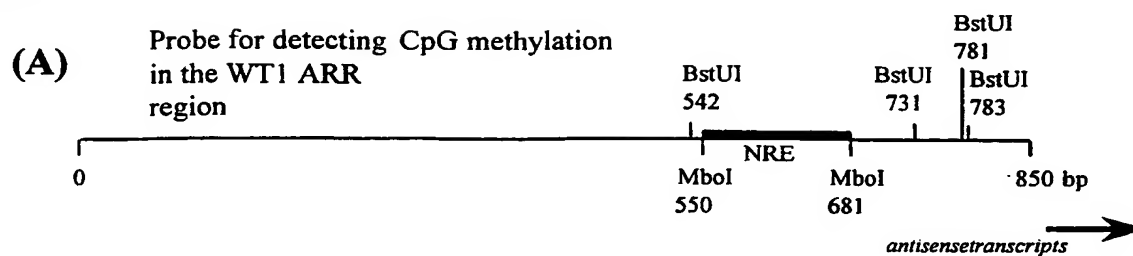
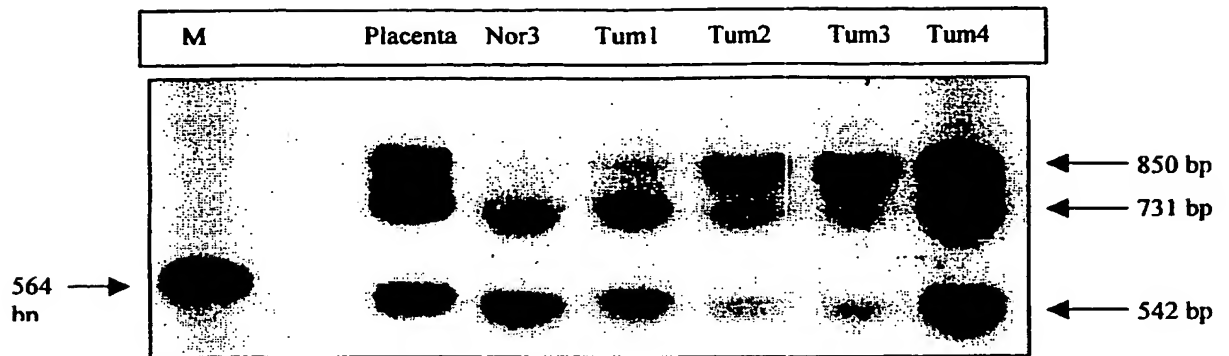
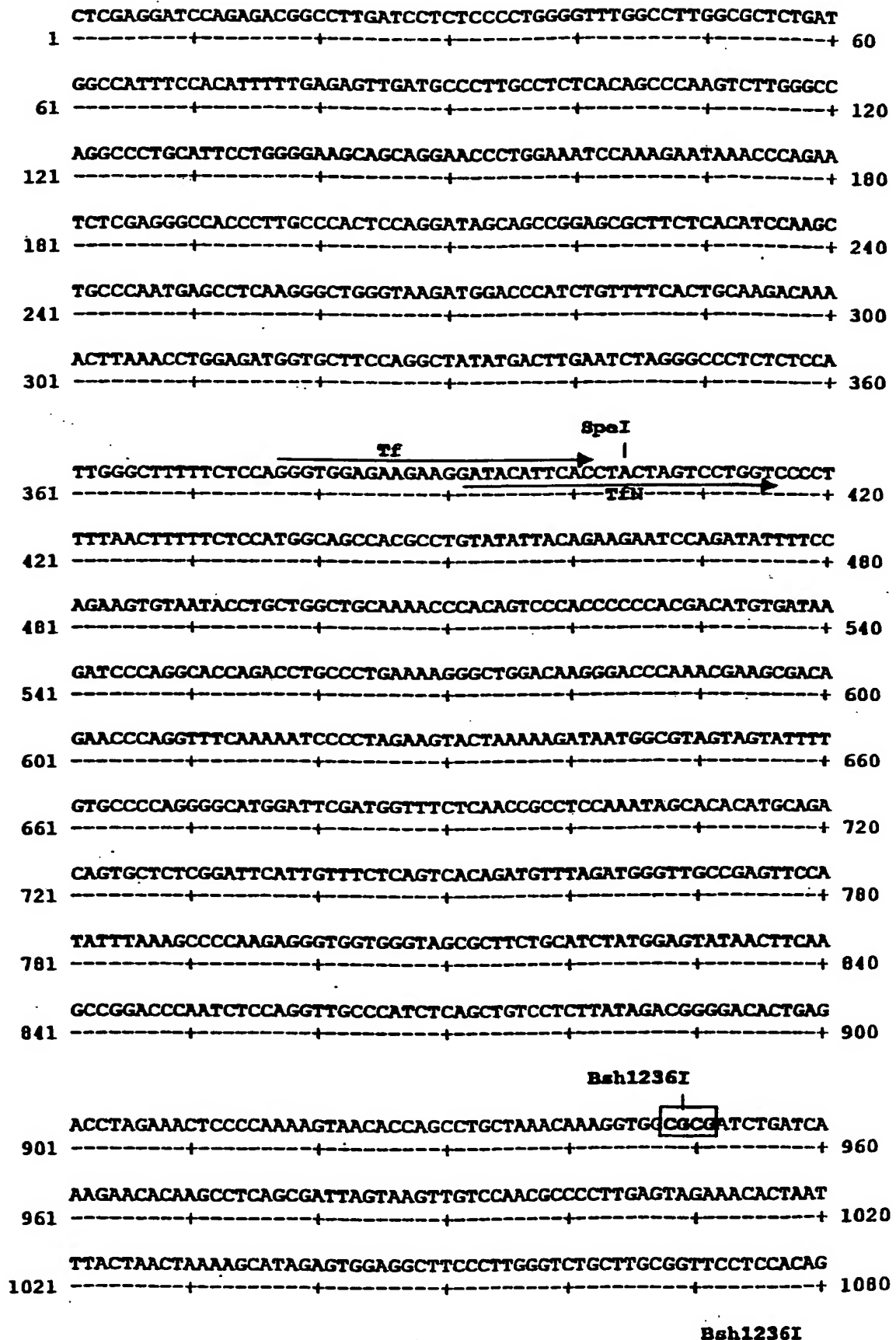


FIGURE 1(E).

M = marker DNA, Nor = normal breast tissue, Tum = breast tumour tissue

Fig.2



1081 GACAGTGATCCCAGATTCTCCCGAAGAAAAGGGCGGTTTCGATTCTCCAAGGCTTCGCG 1140

Bsh1236I
Bsh1236I |

1141 GGGGCCGGGTGCTCCTGGTTAAACTAAGGTAGGAGCGGCCTGAAGACGCGCGTTTAGAAG 1200

KpnI
|

1201 GCGCCGGGTGAAGCGGGCAACAAGGCAGAGCCCTTCTCCGAGCCTTGGGCGAAGGTAC 1260

1261 CTCCTGCAAAAGATACACTCTGCTTCCCACGCATTCCAAAACATCCCGGTCCCTAGGCC 1320

1321 CTCGAGTAATTTTGCTCCAGGAAAAGCATCCGCCATTGTATTAGTAAAGCGTTTACTAAA 1380

1381 TTACCGAATCAAACCGAACTGGCTTAGGTTCTCAATAGCGTGGAATCCACTGAAAATAA 1440

Bsh1236I

TrN
←

1441 ATGAAGAGGGCAAACCTACAGGGGCTCCGCAGGTTCCGGTCCGCGCCGCCAGGCGAAAGA 1500

Bsh1236I

Tr
←

1501 GAGGTGGGCGGGCATCGGCGCGGGATGAGAAACCAACCTGATACTTATCGTGTGCCGAGT 1560

1561 TCCCTCCTTGTATCCTGACTAAGCACAGCGAATAACCCTGTCCTTGTCTAACCCCAGGT 1620

Bsh1236I

1621 CTTGAAGAAATACTGTCCCAGCTGAGCCCCGCGTTTACAAGATGAAGAGGCGCCCCAGAT 1680

1681 GCGCTGAAAGAAAGGCCAAAGCTCGTGCCTCCTTCCACTGCCTGCGGTAGAACCTGGTCC 1740

1741 CGCATAGCTTGAATCGGATAAGTCAAGTTCTCTTCATCCCAGAACCTGCGTGGCCGC 1800

1801 CGCCTGAGCGAAGCCCAGTGAAGATCCACTTCTGTATTACCATACGGGGG 1850

FIG. 3(A)

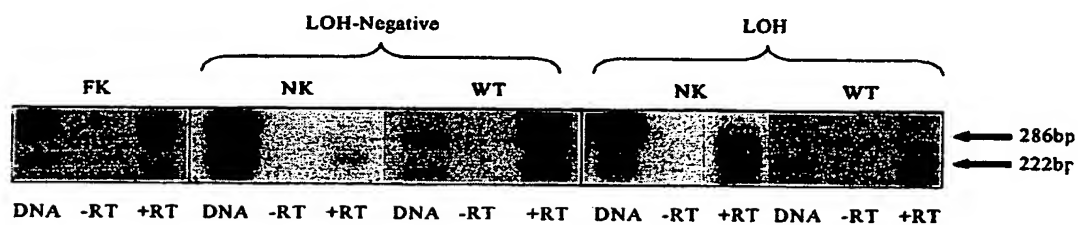
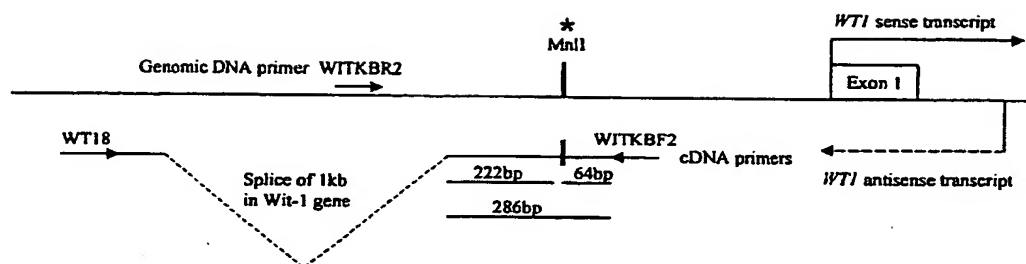


FIG. 3(B)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
25 January 2001 (25.01.2001)

PCT

(10) International Publication Number
WO 01/06005 A3

- (51) International Patent Classification⁷: C12Q 1/68, C07K 14/47, C12N 15/11
- (21) International Application Number: PCT/GB00/02741
- (22) International Filing Date: 17 July 2000 (17.07.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
9916669.6 15 July 1999 (15.07.1999) GB
9926293.3 5 November 1999 (05.11.1999) GB
- (71) Applicant (*for all designated States except US*): **THE UNIVERSITY OF BRISTOL** [GB/GB]; Senate House, Tyndall Avenue, Bristol BS8 1TH (GB).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): **MALIK, Karim** [GB/GB]; Clic Unit, Dept. of Pathology, School of Medical Sciences, University Walk, Bristol BS8 1TD (GB). **BROWN, Keith** [GB/GB]; Clic Unit, Dept. of Pathology, School of Medical Sciences, University Walk, Bristol BS8 1TD (GB).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— *With international search report.*
- (88) Date of publication of the international search report:
25 May 2001
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: DIAGNOSTIC METHOD COMPRISING WT1 SEQUENCES

(57) Abstract: This invention provides a method of detection of cancer in a subject by detecting altered genomic imprinting and a method determining the long term prognosis of a subject diagnosed with cancer, using the differential methylation state of a specific nucleotide sequence to predict the long term prognosis.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT 00/02741

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68 C07K14/47 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, STRAND, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MALIK K T A ET AL: "IDENTIFICATION OF AN ANTISENSE WT1 PROMOTER IN INTRON 1: IMPLICATIONS FOR WT1 REGULATION" ONCOGENE, GB, BASINGSTOKE, HANTS, vol. 11, 1995, pages 1589-1595, XP002910386	1-3, 5, 6, 18, 19
Y	ISSN: 0950-9232 the whole document	7-15, 20-29, 31-37

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

22 February 2001

Date of mailing of the international search report

06/03/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Knehr, M

PCT 00/02741

Relevant to claim No.	
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INTERNATIONAL SEARCH REPORT

International Application No

PC 00/02741

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOORWOOD K ET AL.: "Definition of a novel negative regulatory element of the WT1 antisense promotor" ANTICANCER RESEARCH, vol. 18, no. 6C, 1998, pages 4909-4910, XP000979768 abstract ---	
A	MOORWOOD K ET AL.: "Antisense WT1 transcription parallels sense mRNA and protein expression in fetal kidney and can elevate protein levels in vitro" JOURNAL OF PATHOLOGY, vol. 185, 1998, pages 352-359, XP000981275 cited in the application the whole document ---	
A	US 5 350 840 A (ITO CARYN Y ET AL) 27 September 1994 (1994-09-27) cited in the application the whole document ---	
A	GESSLER M AND BRUNS G A P: "Sequence of the WT1 upstream region including the Wit-1 gene" GENOMICS, vol. 17, 1993, pages 499-501, XP000981270 cited in the application the whole document ---	
A	CAMPBELL C E ET AL.: "Antisense transcripts and protein binding motifs within the Wilms tumour (WT1) locus" ONCOGENE, vol. 9, 1994, pages 583-595, XP000981271 cited in the application the whole document ---	
P,X	MALIK K ET AL.: "Identification and differential methylation of the WT1 antisense regulatory region and relaxation of imprinting in Wilms' tumor" CANCER RESEARCH, vol. 60, 2000, pages 2356-2360, XP002161165 the whole document -----	1-37

FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

Continuation of Box I.2

Claims Nos.: 38

Present claim 38 relates to a method defined by reference to a desirable characteristic or property, namely non-predictable results deriving from non-defined methods. Lacking any support by disclosing no technical feature at all, a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claim also lacks clarity (Article 6 PCT).

Consequently, no search has been carried out for this claim which does not appear to be clear, supported and disclosed.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC 00/02741

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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